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Genetic Basis of the Polymorphisms of the αI Domain of Spectrin

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Defects of α spectrin have been identified in many cases of hereditary elliptocytosis (HE) and hereditary pyropoikilocytosis (HPP). To aid in the genetic analysis of families with these disorders, the locations of three α-spectrin gene polymorphisms were mapped, the genetic basis of these polymorphisms identified, and PCR-based assays designed for their identification. The frequencies of these polymorphisms were determined in two populations and in patients with αI/50a HE and HPP. These studies identified two distinct haplotypes and provided evidence that two HE/HPP mutations associated with the αI/50a protein phenotype, L207P and L260P, arose on separate chromosomal backgrounds. Am. J. Hematol. 56:107–111, 1997. © 1997 Wiley-Liss, Inc.

Key words: spectrin; polymorphism; elliptocytosis; haplotype

INTRODUCTION

The erythrocyte membrane skeleton is responsible for maintaining the shape and deformity of the erythrocyte. Qualitative and quantitative disorders of membrane skeleton proteins have been associated with abnormalities of red blood cell (RBC) shape, including hereditary elliptocytosis (HE) and hereditary pyropoikilocytosis (HPP) [1–4]. HE, characterized by the presence of elliptically shaped erythrocytes in the peripheral blood, is a heterogeneous group of disorders ranging in clinical severity from the asymptomatic carrier state to severe, symptomatic, hemolytic anemia. HPP is an uncommon, severe hemolytic anemia with a characteristic erythrocyte morphology that is reminiscent of that seen in patients after a thermal burn. A number of structural abnormalities of spectrin, the principal component of the erythrocyte membrane skeleton, have been associated with HE and HPP [5–7].

Spectrin is composed of two structurally similar but nonidentical proteins, α and β spectrin. α and β spectrin are composed primarily of homologous 106-amino acid repeats that fold into three antiparallel α-helices connected by short nonhelical segments [8–9]. α and β spectrin combine to form heterodimers, which in turn self-associate to form tetramers and higher order oligomers. These tetramers and oligomers form a lattice-like structure that is critical for erythrocyte membrane stability as well as erythrocyte shape and deformability. Abnormalities of α spectrin associated with HE and HPP are usually due to mutations in the NH_2-terminus of α spectrin, the αI domain, the region of α spectrin that participates in spectrin heterodimer self-association [5–7].

It is useful to identify genetic markers in the α-spectrin gene to aid in the analyses of patients with these disorders. We have precisely mapped the location of three previously described restriction fragment length polymorphisms, *Xba*I, *Msp*I, and *Pvu*II, in the region of the α-spectrin gene encoding the αI domain of spectrin [10]. These polymorphisms are located in introns and are not related to polymorphisms detected by tryptic mapping of spectrin. The genetic basis of these polymorphisms was determined and polymerase chain reaction (PCR)-based assays developed for their analysis. Using these PCR-based assays, the frequencies of these polymorphisms

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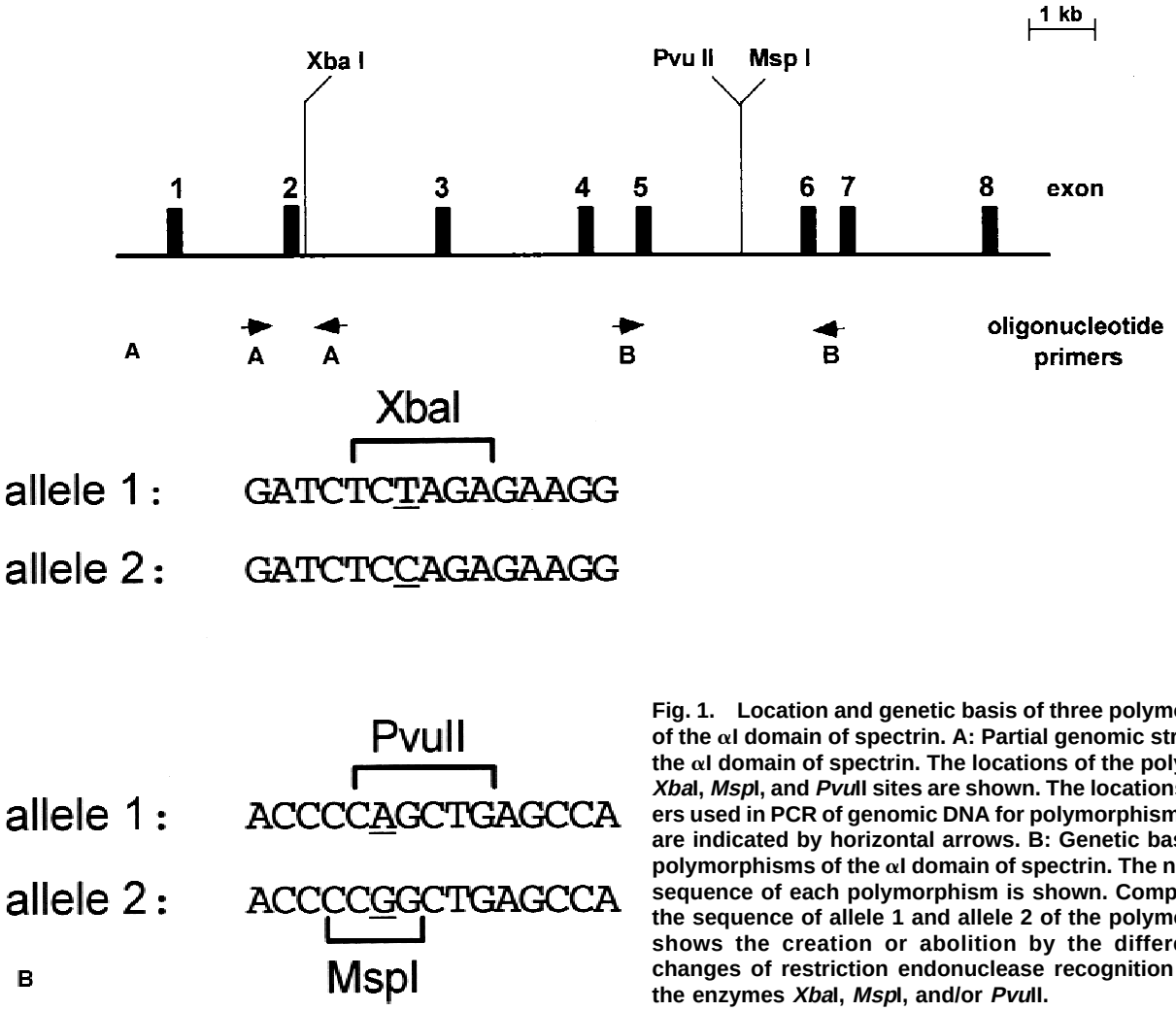

Fig. 1. Location and genetic basis of three polymorphisms of the αI domain of spectrin. A: Partial genomic structure of the αI domain of spectrin. The locations of the polymorphic *Xba*I, *Msp*I, and *Pvu*II sites are shown. The locations of primers used in PCR of genomic DNA for polymorphism analysis are indicated by horizontal arrows. B: Genetic basis of the polymorphisms of the αI domain of spectrin. The nucleotide sequence of each polymorphism is shown. Comparison of the sequence of allele 1 and allele 2 of the polymorphisms shows the creation or abolition by the different base changes of restriction endonuclease recognition sites for the enzymes *Xba*I, *Msp*I, and/or *Pvu*II.

were determined in two populations and in patients with αI/50a hereditary elliptocytosis and pyropoikilocytosis. These studies identified two distinct haplotypes and provided evidence that two HE/HPP mutations associated with the αI/50a protein phenotype, L207P and L260P, arose on separate chromosomal backgrounds.

MATERIALS AND METHODS

Mapping of a Human α-Spectrin Gene Genomic DNA Clone

A recombinant bacteriophage λ clone containing human genomic DNA corresponding to the αI domain of the α-spectrin gene was isolated from a library of human genomic DNA, partially digested with *Eco*RI, using a synthetic α-spectrin gene fragment as a screening probe.

Mapping

Mapping of genomic DNA by restriction enzyme digestion, Southern blot analysis, and hybridization was carried out according to standard techniques. Restriction endonucleases were purchased from Boehringer Mannheim (Indianapolis, IN).

Oligonucleotide Primers for Polymerase Chain Reaction

Synthetic oligonucleotides were synthesized using an automated synthesizer (Applied Biosystems, Inc., Foster City, CA) and purified by OPC column chromatography (Applied Biosystems). The location of oligonucleotide primers used in PCR amplification of the αI domain of spectrin is shown in Figure 1. The nucleotide sequences of these primers were as follows:

primer A (sense): 5'-CGTGAATTCTGAGAACTAGC A ATTAACAG-3'
primer A (antisense): 5'-CGTGGATCCCCATTAACATT AACATAAAG-3
primer B (sense): 5'-GCGAATTCCTGGGAATGCAA GCAGGAGT-3'
primer B (antisense): 5'-AGAGCCTAATACAAAGAC-3'

PCR Amplification of Genomic DNA

Each PCR reaction contained 0.01–0.1 μg of genomic DNA in a 50-μl mixture containing 33 ng of each oligonucleotide primer. Amplifications were carried out for 30 cycles in an automated DNA thermal cycler (model 9600; Perkin-Elmer, Roche Molecular Systems, Branchburg, NJ). Each cycle consisted of 30 sec at 94°C, 1 min at 55°C, and 2 min at 72°C, with the last cycle followed by an additional 5 min of extension time at 72°C.

Subcloning of Amplified DNA

Amplification products were subcloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA) using T4 DNA ligase and transformed in competent *Escherichia coli* as directed (TA cloning kit, Invitrogen, La Jolla, CA).

Nucleotide Sequencing of Subcloned, Amplified DNA

Subcloned, PCR-amplified genomic DNA fragments were sequenced using T7 DNA polymerase (Sequenase, United States Biochemical Corp., Cleveland, OH) and the dideoxy chain termination method of Sanger et al. [11].

Patients and Controls

References detailing the clinical and biochemical characteristics of HE and HPP patients with the αI/50a protein phenotype have previously been described [7]. Control genomic DNA was obtained from unrelated individuals from two ethnic groups: non-HE African-Americans (many with various hemoglobinopathies) and Caucasians of Northern European ancestry.

RESULTS

The isolation of the α-spectrin clone λ3021 from a Charon 4a library of partially *Eco*RI-digested human genomic DNA has been previously described [12]. The genomic structure of the 5′ end of the α-spectrin gene has been previously described [13]. The locations of the *Xba*I, *Msp*I, and *Pvu*II polymorphisms were mapped to the locations shown in Figure 1A. These data agree with that of Lecomte et al. [14] who previously localized the *Xba*I polymorphism to the indicated position in intron 2. Restriction enzyme mapping placed the *Msp*I and *Pvu*II polymorphisms very close together.

Nucleotide sequencing of subcloned fragments of genomic DNA identified the precise genetic basis of these polymorphisms (Fig. 1B). In previous studies, all patients homozygous for the presence of the *Msp*I polymorphism were homozygous for the lack of the *Pvu*II polymorphism [15]. Nucleotide sequencing identified the reason for this observation: a single polymorphic nucleotide is the basis for both polymorphisms. Whenever a C is present (Fig. 1B), an *Msp*I site is created and a *Pvu*II site is abolished. If an A is present, a *Pvu*II site is created and the *Msp*I site is abolished.

Polymerase chain reaction (PCR)-based assays were developed for the analysis of these polymorphisms. Oligonucleotide primers flanking these polymorphisms were used to amplify genomic DNA by PCR. Amplification products were digested with the appropriate restriction enzyme, *Xba*I, *Msp*I, or *Pvu*II, and subjected to agarose gel electrophoresis.

Amplification of genomic DNA by PCR using primer pair A yielded a band of 360 bp. Digestion of amplification products with *Xba*I followed by electrophoresis in a 3% agarose gel identified fragments of 360 bp (uncut) or 330 bp + 30 bp (Fig. 2). Amplification of genomic DNA by PCR using primer pair B yielded a fragment of 2.5 kb. Digestion of amplification products with either *Msp*I or *Pvu*II followed by electrophoresis in a 1.5% agarose gel identified fragments of 2.5 kb (uncut) or 1.4 kb + 1.1 kb (Fig. 2).

One hundred eight alleles of unrelated individuals from two different genetic backgrounds were analyzed by this PCR-based technique (Table I). The frequencies observed are comparable to those previously reported by Hoffman et al. [10] using genomic Southern blots hybridized to a single-copy probe from the λ3021 α-spectrin genomic clone. Co-dominant segregation of these polymorphisms was observed in three informative three-generation families.

These studies identified two allelic haplotypes. In all cases, individuals who were homozygous for digestion with *Xba*I, were also homozygous for digestion with *Msp*I and for lack of digestion with *Pvu*II. The converse was also true: all individuals who were homozygous for lack of digestion with *Xba*I were also homozygous for lack of digestion with *Msp*I and for digestion with *Pvu*II. We propose that the *Xba*I (−)/*Pvu*II (+)/*Msp*I(−) allele (GG*T*CGAC at the *Pvu*II/*Msp*I site) be considered haplotype 1 and the *Xba*I (+)/*Pvu*II (−)/*Msp*I (+) allele (GG*C*CGAC at the *Pvu*II/*Msp*I site) be considered haplotype 2. Because the *Xba*I site and the *Pvu*II/*Msp*I site are > 5 kb apart, crossover could theoretically occur, resulting in individuals with genetic haplotypes that cannot be classified as haplotype 1 or haplotype 2. Mapping and partial nucleotide sequence analysis of the λ3021 α-spectrin genomic clone identified it as haplotype 1.

In many patients with HE and HPP, limited tryptic digestion of spectrin followed by two-dimensional gel electrophoresis has demonstrated abnormal proteolytic cleavage of the 80 kDa peptide of the αI domain of spectrin. Frequently, mutations are found near the site of abnormal proteolytic cleavage [5,16,17]. In HE/HPP patients with one of these proteolytic variants, αI/50a, three different α-spectrin mutations have been identified: L207P, L260P, and S261P [7]. The L207P and L260P

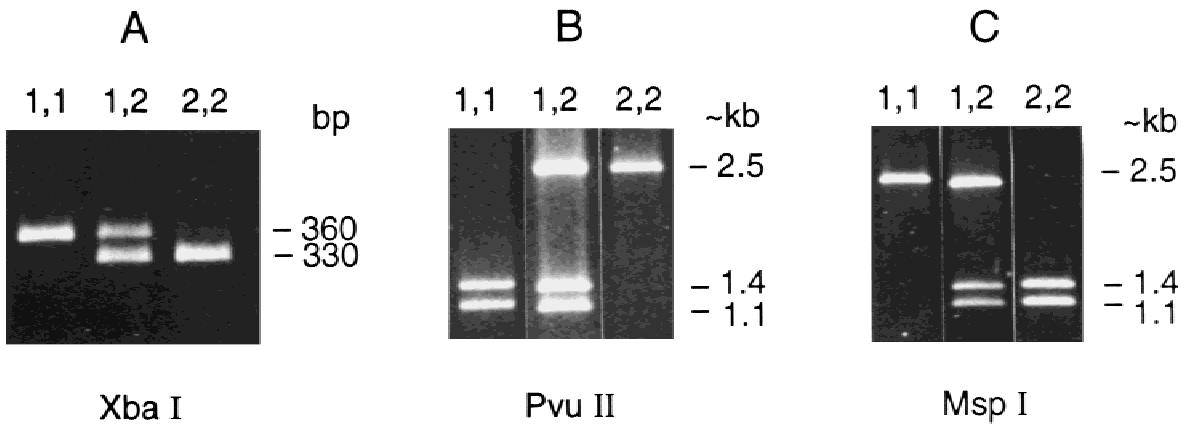

Fig. 2. Analysis of three polymorphisms of the human erythrocyte α-spectrin gene using PCR-based assays. PCR-amplified DNA from three individuals was digested with the restriction endonuclease, *Xba*I, *Pvu*II, or *Msp*II, respectively, and fractionated by agarose gel electrophoresis. A and C: The DNA in lane 1 lacks the polymorphism (–,–); the DNA in lane 2 is heterozygous for the polymorphism (+,–); and the DNA in lane 3 is homozygous for the polymorphism (+,+). B: Digestion of DNA from the same three individuals with *Pvu*II shows that the DNA is lane 1 is homozygous for the polymorphism (+,+), the DNA in lane 2 is heterozygous for the polymorphism (+,–) and the DNA in lane 3 lacks the polymorphism (–,–). This confirms the *Xba*I/*Msp*I/*Pvu*II haplotypes of –,+,– and +,–,+, respectively (see text for details).

TABLE I. Allelic Frequencies of Three α-Spectrin Gene Polymorphisms*

Restriction endonuclease	Frequency		Fragment sizes of PCR products
	Caucasians	African-Americans	
*Xba*I	Allele 1: 0.53	Allele 1: 0.47	360 bp
	Allele 2: 0.47	Allele 2: 0.53	330 + 30 bp
*Pvu*II	Allele 1: 0.47	Allele 2: 0.53	1.4 + 1.1 kb
	Allele 2: 0.53	Allele 2: 0.47	2.5 kb
*Msp*I	Allele 1: 0.53	Allele 1: 0.47	2.5 kb
	Allele 2: 0.47	Allele 2: 0.53	1.4 + 1.1 kb

*No. of Caucasians = 58; no. of African-Americans = 60.

TABLE II. Correlation of Haplotypes and α-Spectrin Mutations in Patients With Hereditary Elliptocytosis and Hereditary Pyropoikilocytosis

Mutation	αI domain haplotype[a]	αII domain haplotype[b]
L154Ins	2	1
L207P	2	1
L260P	1	2
S261P	1	1

[a]Defined in Materials and Methods.
[b]Defined in reference 18.

mutations are common in HE/HPP patients of African descent [2]; the S261P mutation has been identified only in a single Hispanic patient [13]. We analyzed the αI domain polymorphisms in twelve unrelated individuals with αI/50a HE or HPP due to the L207P mutation (7 patients), the L260P mutation (4 patients), or the S261P mutation (1 patient). The distance of these mutations in genomic DNA from the *Msp*I/*Pvu*II polymorphic site is close enough to allow amplification of DNA fragments containing both the mutation and the polymorphic site in a single PCR reaction from each individual. Amplification products were subcloned and individual subclones analyzed for the presence or absence of the mutation and polymorphism. Examination of the data reveals that there are common allelic haplotypes for each mutation, haplotype 1 for the L260P and S261P mutations (4 patients and 1 patient, respectively) and haplotype 2 for the L207P mutation (7 patients) (Table II).

DISCUSSION

These studies demonstrate that the αI domain polymorphisms of spectrin exhibit marked allelic variability in two different populations, making them useful in genetic linkage studies of kindreds with HE and HPP. It also shows that it is unnecessary to perform PCR-based assays for all three polymorphisms to ascertain the status of the αI domain chromosomal haplotype. Excluding crossover between the *Xba*I and the *Pvu*II/*Msp*I polymorphisms which can occur very rarely: (A.-F. Roux, PhD Thesis, University Claude Bernard-Lyon I, 1990), analysis of a single polymorphism should provide sufficient data to deduce the status of the other sites; at the most, *Xba*I and either *Pvu*II or *Msp*I are all that are necessary for determination of a haplotype.

The identification of these polymorphisms permits amplification of a single DNA fragment containing both an αI domain polymorphism and an αI domain HE/HPP mutation, allowing correlation of the polymorphism (and the haplotype) with the mutation, particularly in heterozygous individuals. This is true for αI/50a mutations that occur in either exon 5 or 6, which flank the *Msp*I/*Pvu*II sites, and for αI/74 mutations occurring in exon 2 immediately 5′ of the *Xba*I site [5,13]. This will facilitate epidemiologic and population-based genetic studies of HE and HPP mutations in the α-spectrin gene.

The finding of separate allelic haplotypes for each of the two common αI/50a HE/HPP mutations suggests the presence of a ''founder effect'' for each mutation, i.e., these two mutations arose on separate chromosomal backgrounds. This finding corroborates haplotyping studies of the αII domain polymorphisms of spectrin [18]. The L207P mutation was found only on an αII domain-haplotype 1 allele, whereas the L260P mutation was found only on an αII domain-haplotype 2 allele. Together, these data suggest that the L207P mutation arose on an α-spectrin allele of the αI-haplotype 2/αII-haplotype 1, and that the L260P mutation arose on an α-spectrin allele of the αI-haplotype 1/αII-haplotype 2.

These data also corroborate the epidemiological studies of HE-related spectrin mutations and α-spectrin gene polymorphisms in Benin, Africa [19]. The L260P mutation was found in a restricted area of southern Benin among two very closely related ethnic groups, the Fon and the Yoruba. The term ''Nigerian allele'' was proposed to describe an α-spectrin gene allele containing the L260P HE mutation, the αII-haplotype 2, and two other α-spectrin gene polymorphisms/mutations, R1331I and R1496W. We propose that the αI-haplotype 1 be an additional descriptor of the Nigerian allele.

In a similar manner, it should be noted that the common αI/68 HE phenotype associated with a leucine insertion, L154Ins, only arises on the αI-haplotype 2 α-spectrin gene chromosomal background [15,20].

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